The *BAX* Gene, the Promoter of Apoptosis, Is Mutated in Genetically Unstable Cancers of the Colorectum, Stomach, and Endometrium[1]

Hong Ouyang, Toru Furukawa, Tadayoshi Abe, Yo Kato, and Akira Horii[2]

Department of Molecular Pathology, Tohoku University School of Medicine, Sendai 980-8575 [H. O., T. F., T. A., A. H.], and Department of Pathology, Cancer Institute, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170 [Y. K.], Japan

ABSTRACT

Disruption of the DNA mismatch repair system, characterized by microsatellite instability (MI), plays an important role in the course of human carcinogenesis by increasing the rate of mutations of genes associated with cancers. However, it is not clear which genes are the target genes for mutation in the course of carcinogenesis. Microsatellites within the coding region of the transforming growth factor β receptor type II (*RII*) and insulin-like growth factor II receptor (*IGF-IIR*) genes were reported to be targets for mutation during the course of carcinogenesis in MI+ tumors. Recently, somatic mutations were found in a poly(G)$_8$ tract in the BCL-2-associated X protein (*BAX*) gene, one of the essential players in apoptosis, in some MI+ tumors. We examined mutations of *BAX* in MI+ cancers of various organs and found frameshift mutations at the poly(G)$_8$ tract in 5 of 15 (33%) gastric cancers, 3 of 26 (12%) endometrial cancers, and 9 of 22 (41%) colorectal cancers. In contrast, no such mutations were found in pancreatic cancer. These results suggest that mutations of *BAX* play an important role in the course of carcinogenesis in the stomach, colorectum, and endometrium.

INTRODUCTION

MI,[3] or instability in simple repeated sequences, has been associated with HNPCC as well as several sporadic forms of human cancer (1–4). This behavior is thought to be caused by disruption of the mismatch repair system, leading to increased rates of mutation within genes, some of which presumably are cancer related or even cancer causing. Hence, it is very important to determine the target gene(s) for mutations in MI+ cells. Somatic mutations in the poly(A)$_{10}$ and poly(G)$_8$ tracts in the coding regions of the *RII* and *IGF-IIR* genes, respectively, have been reported in gastric, colorectal, and endometrial cancers with MI (5–10). Recently, mutations in the poly(G)$_8$ tract of a third gene, *BAX*, were reported in some MI+ cell lines and primary colon tumors (11). BAX is a BCL-2-related protein that counteracts BCL-2 and promotes apoptosis (12). An increasing amount of evidence has indicated that the expression of the *BAX* gene parallels p53-mediated apoptosis (13). Moreover, the *BAX* gene was found to be directly activated by p53 (14). Thus, BAX is thought to be required for p53-mediated apoptosis (15). In this connection, it is of great interest to examine whether or not genes such as *BAX* that are associated with apoptosis are somatically mutated. Additionally, interleukin-1β converting enzyme (ICE) is also reported to play a key role in apoptosis (16); this gene also contains a poly(A)$_8$ tract in the coding region (17). Thus, *ICE* is also one of the candidates for mutation in MI+ tumors. Herein, we report mutational analyses of the *BAX* and *ICE* genes in gastric, colorectal, pancreatic, and endometrial cancers, a group with a high incidence of MI.

MATERIALS AND METHODS

Materials and DNA Extraction. A total of 360 paired tumors and corresponding normal tissues from Japanese patients at Tohoku University Hospital and its related hospitals (Sendai, Japan) and Cancer Institute Hospital (Tokyo, Japan) were analyzed. These samples are listed in Table 1. DNA was extracted according to methods described previously (8). Histopathological diagnoses were classified according to the WHO criteria for colorectal and endometrial cancers (18, 19), the WHO and Lauren's criteria for gastric cancer (20, 21), and the JPS criteria for pancreatic cancer (22). Clinical stages were determined according to Dukes' criteria for colon cancer, the International Federation of Gynecologists and Obstetricians criteria for endometrial cancer (23), the Japanese Research Society for Gastric Cancer criteria for gastric cancer (24), and the JPS criteria for pancreatic cancer (22). The JPS and Japanese Research Society for Gastric Cancer criteria for gastric and pancreatic cancers were summarized in our previous reports (10, 25).

Analyses of MI. MI was determined using five or more of the microsatellite markers. Nucleotide sequences and detailed conditions for PCR amplifications are available upon request. In each case, one of the primers was ^{32}P end-labeled, and paired DNAs of normal and cancerous tissues were amplified by PCR followed by electrophoresis in 6% polyacrylamide/8 M urea/32% formamide gels as described previously (8). Tumors in which altered-sized bands were observed at two or more (or 40% or more) of the microsatellite loci were defined as MI+.

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[2] To whom requests for reprints should be addressed, at Department of Molecular Pathology, Tohoku University School of Medicine, Sendai 980-8575, Japan. Phone: 81-22-717-8042; Fax: 81-22-717-8047; E-mail: horii@mail.cc.tohoku.ac.jp.

[3] The abbreviations used are: MI, microsatellite instability; BAX, BCL-2-associated X protein; ICE, interleukin-1β converting enzyme; IGF-IIR, insulin-like growth factor II receptor; RII, transforming growth factor β receptor type II; HNPCC, hereditary nonpolyposis colorectal cancer; JPS, Japan Pancreas Society.

Table 1 Summary of mutations detected in *BAX*

Tumors	Histological diagnosis and clinical stage[a]	No. of tumors	No. of MI+ cases	No. of *BAX* mutations
Endometrial cancer		100	26	3
	G_1	48	11	1
	G_2	29	8	1
	G_3	20	6	1
	Clear	2	1	0
	Serous	1	0	0
	Stage I	65	19	2
	Stage II	16	3	0
	Stage III	18	4	1
	Stage IV	1	0	0
Gastric cancer		82	15	5
	Intestinal	35	7	2
	Diffuse	38	4	1
	Others	9	4	2
	Stage I	27	5	1
	Stage II	12	3	1
	Stage III	32	5	2
	Stage IV	11	2	1
Colorectal cancer[b]		118	22	9
	Well	40	4	1
	Mod	41	0	0
	Poor	33	17	7
	Muc	4	1	1
	Dukes' A	51	8	2
	Dukes' B	10	2	1
	Dukes' C	45	9	5
	Dukes' D	12	3	1
Pancreatic cancer		60	9	0

[a] G_1, endometrioid adenocarcinoma grade 1; G_2, endometrioid adenocarcinoma grade 2; G_3, endometrioid adenocarcinoma grade 3; clear, clear cell adenocarcinoma; serous, serous adenocarcinoma; intestinal, intestinal type of gastric cancer; diffuse, diffuse type of gastric cancer; well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma; muc, mucinous adenocarcinoma.

[b] Two of the 118 colorectal cancers were developed in 1 HNPCC patient.

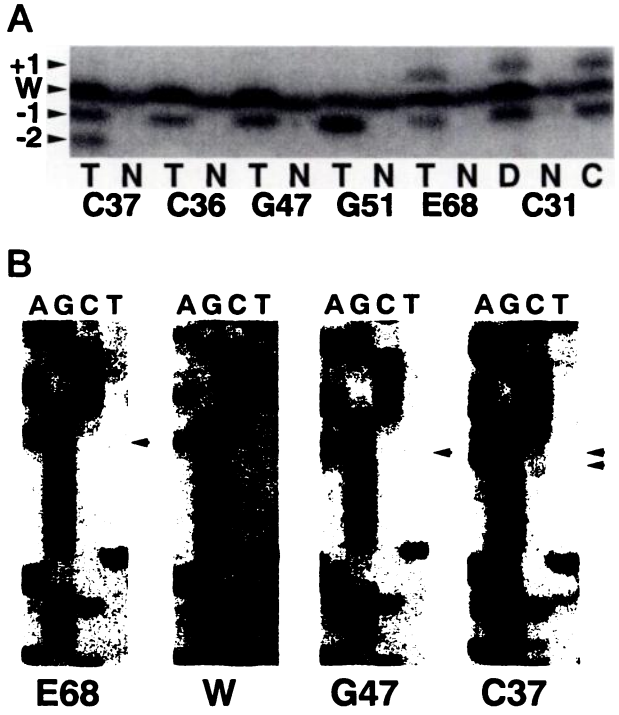

Fig. 1 *A*, screening for mutations of the poly$(G)_8$ tract (an eight-deoxyguanine repeat) in the *BAX* gene. Deletions of 1 bp in a colorectal cancer (*C36*) and gastric cancers (*G47* and *G51*) are shown. Both the 1-bp deletion and the 1-bp insertion were shown in descending colon (*D*) and cecum (*C*) cancers of one HNPCC patient and in endometrial cancer case E68, whereas the 1- and 2-bp deletions were observed in a colorectal cancer (*C37*). *T* and *N* denote DNAs from tumors and the corresponding normal tissues, respectively. *W*, *+1*, *−1*, and *−2* denote the wild type, 1-bp insertion, and 1- and 2-bp deletions, respectively. *B*, nucleotide sequencing analysis at the poly$(G)_8$ tract of the *BAX* gene in *E68* (endometrial cancer), *G47* (gastric cancer), and *C37* (colorectal cancer). Nucleotide sequences of the sense strands are shown. Deletions of 1 and 2 bp and an insertion of 1 bp at the poly$(G)_8$ tract are indicated by *arrowheads*. The nucleotide sequence of the wild type (*W*) is also shown.

Mutation Analyses of *BAX* and *ICE*. Mutations of *BAX* and *ICE* at the repetitive sequences within the coding regions were determined by a PCR-based assay (10). In brief, each 15-μl reaction mixture [10 ng of DNA, 6.7 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 6.7 μM EDTA, 6.7 mM $MgCl_2$, 0.33 mM of [γ-^{32}P]ATP-labeled and unlabeled primer, 1.5 mM each deoxynucleotide, 10% (v/v) DMSO, and 0.75 unit of Taq DNA polymerase] was amplified for 40 cycles with the following regime: denaturation at 94°C for 30 s; annealing for 30 s at 55°C for the poly$(C)_6$ tract of *BAX* and the poly$(A)_8$ tract of *ICE* and at 58°C for the poly$(G)_8$ tract of *BAX*; and extension at 72°C for 30 s. Repetitive sequences within the coding region of the *BAX* gene [the poly$(G)_8$ and poly$(C)_6$ tracts at nucleotides 114–121 and 260–265, respectively] and the *ICE* gene [the poly$(A)_8$ tract at nucleotides 2084–2091] were analyzed. Nucleotide sequences of the primers were as follows: 5′-TTCATCCAGGATCGAGCAGG-3′ and 5′-ACTCGCTCAGCTTCTTGGTG-3′ for the poly$(G)_8$ tract in *BAX*; 5′-ATGATTGCCGCCGTGGACA-3′ and 5′-CAGTTGAAGTTGCCGTCAGA-3′ for the poly$(C)_6$ tract in *BAX*; and 5′-ATCTGGGGTACAGCGTAGAT-3′ and 5′-TAGAACTTACCGAAGCAGTGA-3′ for the poly$(A)_8$ tract in *ICE*. Mutations were confirmed by nucleotide sequencing analysis according to the methods described previously (10).

RESULTS

We first examined *BAX* for mutations at repetitive sequences within its coding region in 360 human tumors of 4 different organs, as summarized in Table 1. Two regions of the gene, an eight-guanine stretch [the poly$(G)_8$ tract at nucleotides 114–121] and a six-cytosine stretch [the poly$(C)_6$ tract at nucleotides 260–265], were analyzed. Typical examples are shown in Fig. 1*A*. Colorectal cancer case C37 showed 1- and 2-bp deletions, whereas C31D and C31C, cancers that had developed in a single HNPCC patient, showed a 1-bp insertion and a 1-bp deletion at the poly$(G)_8$ microsatellite within *BAX*. These tumors had two-hit mutations; the wild-type band was presumably contributed by contaminating normal cells. Similarly, in case E68, an endometrial cancer, a two-hit mutation was observed; a 1-bp insertion as well as a 1-bp deletion was clearly seen. Gastric cancer cases G47 and G51 and colorectal

Table 2 Summary of mutations detected in *BAX*, *IGFIIR*, and *RII*

Tumors	Case no.	Histological diagnosis[a]	Clinical stage	Mutations[b]			
				BAX	*IGFIIR*	*RII*	MI
Colorectal cancer[c]	C23	Well	Dukes' A	−	−	+	+
	C31C	Well	Dukes' A	+	−	+	+
	CA36	Mod	Dukes' A	−	−	+	+
	C1P	Poor	Dukes' C	−	ND	+	+
	C4	Poor	Dukes' C	−	ND	+	+
	C5	Poor	Dukes' D	+	−	+	+
	C7	Poor	Dukes' A	−	ND	+	+
	C8	Poor	Dukes' A	+	ND	+	+
	C10	Poor	Dukes' C	+	−	+	+
	C22	Poor	Dukes' D	−	−	+	+
	C29	Poor	Dukes' B	+	−	+	+
	C31D	Poor	Dukes' C	+	+	+	+
	C36	Poor	Dukes' C	+	−	+	+
	C37	Poor	Dukes' C	+	−	+	+
	C62	Poor	Dukes' A	−	−	+	−
	C63	Muc	Dukes' C	+	−	−	+
Gastric cancer	G28	Intestinal	Stage III	−	−	+	+
	G34	Intestinal	Stage I	−	−	+	+
	G48	Intestinal	Stage I	+	−	+	+
	G84	Intestinal	Stage I	−	+	+	+
	G105	Intestinal	Stage I	−	−	+	+
	G139	Intestinal	Stage III	+	+	−	+
	G1	Diffuse	Stage III	−	−	+	−
	G37	Diffuse	Stage I	−	−	+	+
	G41	Diffuse	Stage IV	−	−	+	−
	G51	Diffuse	Stage III	+	−	−	+
	G18	Others	Stage III	−	−	+	+
	G35	Others	Stage II	+	−	−	+
	G47	Others	Stage IV	+	+	+	+
Endometrial cancer	E87	G_1	Stage I	−	+	−	+
	E115	G_1	Stage I	+	−	−	+
	E517	G_1	Stage I	−	+	−	+
	E510	G_1	Stage II	−	+	−	+
	E90	G_2	Stage I	−	+	−	+
	E94	G_2	Stage I	+	−	−	+
	E68	G_3	Stage III	+	−	−	+

[a] G_1, endometrioid adenocarcinoma grade 1; G_2, endometrioid adenocarcinoma grade 2; G_3, endometrioid adenocarcinoma grade 3; clear, clear cell adenocarcinoma; serous, serous adenocarcinoma; intestinal, intestinal type of gastric cancer; diffuse, diffuse type of gastric cancer; well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma; muc, mucinous adenocarcinoma.

[b] +, mutations were detected; − mutations were not detected; ND, not determined.

[c] Two of the 118 colorectal cancers, C31C and C31D, were developed in 1 HNPCC patient.

cancer case C36 showed 1-bp deletions. These results are summarized in Tables 1 and 2. No mutation was observed in any of the pancreatic cancers.

To confirm that these alterations occurred in the repetitive poly$(G)_8$ region of each PCR product showing mutation, the nucleotide sequences of these tumors together with DNAs from their corresponding normal tissues were analyzed. Examples are shown in Fig. 1*B*. DNA from endometrial cancer E68 gained one guanine residue in the poly$(G)_8$ region of *BAX*, whereas in gastric cancer G47, a 1-bp deletion in the poly$(G)_8$ tract was clearly observed. Similarly, in C37, both 1- and 2-bp deletions were observed. We further subcloned the PCR product of tumor DNA C37 in the *Eco*RV site of pBluescript II SK(+) and determined the nucleotide sequences of the 40 independent clones: the number of clones containing poly$(G)_8$, poly$(G)_7$, and poly$(G)_6$ was 18, 11, and 11, respectively.

We found no mutations in the poly$(C)_6$ tract of the *BAX* gene or in the poly$(A)_8$ tract of the *ICE* gene (data not shown). We further screened MI− cancers in these organs, but again, no mutations were found (data not shown). In conclusion, mutation of the *BAX* gene was observed in three endometrial, five gastric, and nine colorectal cancers with MI. No correlation between *BAX* mutation and age, stage, or histological type was observed. Tumors in which mutations of the *BAX* gene were observed are listed in Table 2 with our previous results for *IGF-IIR* and *RII* (8, 10).

DISCUSSION

Recently, MI has been found to play a significant role in various human tumors (1–4). MI+ tumor cells tend to have mutations in repetitive sequences throughout the genome (26), and frequent mutations were reported in *RII* and *IGF-IIR* in MI+ cancers (8–10). BAX is a BCL-2-related protein that promotes apoptosis. Mutation of *BAX* was reported in cell lines derived from hematological malignancies (27). Recently, frequent frameshift mutations of *BAX* in the microsatellite within

the coding sequence were reported in some cell lines and primary colon tumors (11). In the present study, we observed somatic frameshift mutations in the poly$(G)_8$ microsatellite of *BAX* in MI+ endometrial (3 of 26 cases, 12%), gastric (5 of 15 cases, 33%), and colorectal (9 of 22 cases, 41%) cancers; frameshift mutation should result in the production of truncated BAX protein. Thus, our present results suggest that: (*a*) mutations of the *BAX* gene play an important role in the genesis of endometrial, gastric, and colorectal cancers with MI; and (*b*) the *BAX* gene is one of the target genes for genetic alterations in gastric, colorectal, and endometrial cancers with MI.

Although the incidence of MI is high in pancreatic cancer, we did not observe any mutation in *BAX* in 60 pancreatic cancers, 9 of which (15%) were MI+. We previously studied mutations of *RII* and *IGF-IIR* and found no mutations in pancreatic cancers. There are possible explanations for this finding: (*a*) BAX does not play an important role in pancreatic carcinogenesis; or (*b*) mutations in other regions of the gene are crucial in pancreatic carcinogenesis. Because repetitive sequences as analyzed in this study are frequent targets for mutation in genetically unstable cells, we suspect that the former possibility is more likely than the latter. There may be a gene(s) other than *BAX*, *RII*, or *IGF-IIR* that plays an important role(s) in pancreatic carcinogenesis. Additional studies are necessary to identify other possible target gene(s) of MI in this tumor type to understand the course of carcinogenesis.

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